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by

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The primary objective of this study is to evaluate chemical preservatives which can be added to the sample bottle before collection which will be effective in stabilizing the coliform density for not less than 48 hours using current methodology for detecting coliform organisms. This study evaluates the stability of control samples stored at 22°C and 4°C which are compared to each other and to the stability of test samples which contained different preservatives and preservative concentrations. Candidate preservations were chosen based upon their reported stabilizing effect in foods, bacteriological holding medium and as stabilizing agents used to prepare quality control performance samples.

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SAMPLE PRESERVATION: A PROGRESS REPORT

Harry D. Nash and Edwin E. Geldreich

A solution to sample preservation during transit and storage time has been sought by many investigators for decades with varying and limited degrees of success (2,3,4,6). Coliform density has been shown to very with water chemistry, microbial flora, and time. These variables make it difficult to predict whether or not the change in coliform density will increase or decrease. Identified variables that influence indicator organism survival include: temperature, concentration of organic and inorganic nutrients, dissolved oxygen, toxic substances, non-coliform organisms, recoverability of stressed coliforms and other undefined factors and combination of factors present in waters from widely separated geographical areas. As transit/storage time is prolonged, regardless of ambient temperature conditions, the dynamic interactions of bacteria and the aquatic environment result in changes in bacterial numbers and predominant bacterial types.

Many investigators have contributed information regarding the effects of transit time on water samples. Berry (1) reported that coliform bacteria in well water samples did not change significantly during 48 hours storage at ambient temperatures or when iced. However, Caldwell and Parr (2), also studying well water samples, observed that the coliform group decreased to one-fifth the initial count within a few hours and less than one-tenth when stored from 24 to 30 hours under iced conditions. Uniced samples showed only a 4 percent recovery compared to the initial count. Hoather (3) examined 140 samples from public water supplies three to seven hours and approximately 24 hours after collection. His data indicated a 12 percent decrease in the initial density when the samples were stored at 5°C for 24 hours and even a

smaller decrease was observed for samples stored at 20°C. Hoather's observations were confirmed in another report published by Cox and Clairborne (4) who observed similar results. In contrast, Jones et al. (5) reported that two hours after sampling and again after 18 hours storage at 3° to 5°C, 21 percent of the water samples analyzed decreased significantly from the initial density. Nash and Geldreich (6), studying coliform spiked potable water samples, observed a decrease in coliform recovery after 24 hours and again between 30 and 48 hours storage at 22°C. Although the reduced recovery may have resulted from random distribution of coliforms at an initial coliform density of less than 10/100 mL, in the sample volume examined, a definite trend toward decreased coliform recovery was indicated. They also observed that sample refrigeration appeared to minimize the problem of decreased coliform recovery even after a 54 hour storage period. Standridge and Delfino (7) studied randomly selected samples from those routinely collected throughout Wisconsin which were analyzed for total coliforms after being held at 20 ± 2°C for 24 and 48 hours. Differences in results for the two holding times were compared with differences predicted by probability calculations. They observed that potable water storage up to 48 hours at 20 ± 2°C had little effect on the public health significance of most samples containing more than two coliforms per 100 mL. However, their analyses were only conducted after a 24 hour storage time and data was not available to compare to the initial coliform density at the time of collection. McDaniels and Bordner (8) stated that coliform populations in water samples collected from a distribution system and stored at 5°C and 22°C declined significantly after 24, 30, and 48 hours storage, but not after 2, 6 or 12 hours as measured by both the MF and MPN methods. They reported that unacceptable initial coliform densities

of 5 and 17/100 mL for two samples out of 12 held at 5°C for 24 hours declined to acceptable levels of 4 or less/100 mL as did initial counts ranging from 5 to 31/100 mL for five samples out of 12 held at 22°C. The mean coliform recovery for samples held at 5°C was 66,54, and 54 percent for 24, 30, and 48 hour storage periods, respectively. For samples sorted at 22°C, the recovery was 13, 8, and 5 percent at 24, 30, and 48 hours respectively. They concluded that because of coliform losses during storage, samples with counts that were initially unacceptable could have been reported as acceptable if held for 24 hours at either ambient or refrigerated temperatures. Therefore, a recommendation was made that all drinking water samples be iced and analyzed within six hours of collection.

Numerous comparative studies can be cited indicating inconsistant results relating to the basic problem of maintaining the initial coliform density during some specified storage time and temperature. The concensus that is derived from published studies is that some refrigeration affords the best means of sample preservation, and provides the most reliable data other than on site sample analysis. Unfortunately, sample refrigeration using dry ice, gels, and thermo insulated containers increases the shipping cost due to added weight and is a potential source of sample contamination. Sample preservation, other than refrigeration, would be advantageous with regard to sample transit costs.

The primary objective of this study is to evaluate chemical preservatives which can be added to the sample bottle before collection which will be effective in stabilizing the coliform density for not less than 48 hours using current methodology for detecting coliform organisms. This study evaluates the stability of control samples stored at 22°C and 4°C which are compared

to each other and to the stability of test samples which contained different preservatives and preservative concentrations. Candidate preservations were chosen based upon their reported stabilizing effect in foods, bacteriological holding medium and as stabilizing agents used to prepare quality control performance samples (9,10).

Current Evaluation Protocol

Approximately 30-40 liters of water were collected from a laboratory faucet in a 50-liter, sterile, polypropylene container. This volume was mixed and a minimum of three liters transferred to sterile widemouth one gallon polypropylene containers. This minimized variation of water used for each test sample. Free chlorine residuals were determined with a Hach field kit using N, N- diethylp-phenyldiamine (DPD) colorimetric technique. Sodium thiosulfate at a concentration of 100 mg/L was added to neutralize any free residual chlorine in each test sample. All test samples containing various concentrations of preservative were stored for a maximum of 72 hours at 22°C. Two test samples which contained no preservative were prepared as controls; one was stored at 4°C, and the other at 22°C. One of three coliform organisms Enterobacter cloacae, Klesbiella pneumoniae or Citrobacter freundii, isolated from a water distribution environment was used to inoculate test and control samples. Cultures used to inoculate all samples were grown in 5 mL of tryptic soy broth, incubated for 18 to 24 hours at 35°C, centrifuges, washed three times with sterile phosphate buffer water and the resulting packed cells were resuspended in a 5-mL volume of sterile phosphate buffer water. The cell suspension was diluted and an appropriate inoculum volume was added to yield a coliform concentration between 30 and 80 cells/100 mL.

All test samples were mixed well and analyzed immediately after inocula-

tion to obtain the initial coliform density. Test samples were analyzed again after 24, 48, and 72 hours storage. This procedure was repeated a minimum of five times for each preservative screened. After determining the most promising preservatives by this screening process, the same basic protocol will be used to analyze samples obtained from potable water sampling locations which have had a history of low level indicator organism occurrences.

Statistical Analyses

Several response measures were considered in order to measure the stability of the samples over time:

1. Proportion of initial count = $\frac{\text{Mean count at specific hour}}{\text{mean initial count}}$

2. Mean difference = mean count at specific hour - mean initial count

However, because the distributions of the response variables were skewed, various tranformations were examined and the following was chosen for use in the statistical analysis: log proportion = log (proportion of initial count + 0.1).

A three factor (dose, method, time) repeated measure analysis of variance was conducted. This procedure allows for comparison among various preservative concentrations over time and between methods. A contrast comparison of each concentration of the preservative to the controls was included in the analysis (11). All data points represent at least five individual analyses for each preservative investigated.

Results

A. Boric Acid

The ability of boric acid to function as a preservative and the differences between the membrane filter (MF) single step and enrichment step procedures (12) are shown in Figures 1 & 2. Figure 1 indicates no significant difference in the stability of the coliform counts between the two control samples which

contained no preservative and were stored at 4° and 22°C, respectively. The 22°C control sample appeared to be slightly more stable than the 4°C control during the 48 hour storage period. After a 72 hour storage period, the 22°C control increased while the 4°C control decreased. Neither of the tests samples containing 1.0% and 0.8% boric acid weight to volume (w/v) were significantly more stable than the controls. Data indicate that boric acid, at the concentrations used does not act as a preservative. Figure 2, shows similar data obtained using the enrichment rather than the single step MF procedure and indicates that the difference between the two MF procedures was of borderline significance ($p = 0.06$). The overall average proportional change of the single step procedure counts was slightly less than that for the enrichment procedure counts. Thereafter, parallel sample analysis by both MF procedures was discontinued and the single step MF procedure was utilized for sample analyses.

B. Sodium Chloride (NaCl)

Figure 3 shows the results from the evaluation of sodium chloride as a preservative. No significant difference was observed between the two control samples over time. Of the three concentrations of sodium chloride, 0.05%; 0.1%; and 0.3% (w/v), the two test samples that contained concentrations of 0.05% and 0.1% showed no significant preservative effect relative to the controls. The coliform density of the test sample that contained 0.3% NaCl increased with time as compared to the controls.

C. Sodium Benzoate

Figure 4 shows the results of experiments which assessed sodium benzoate as a preservative. There was no significant difference in counts between the two controls samples over time. The control counts tended to decrease while test sample counts at sodium benzoate concentrations of 0.3% and 0.8% (w/v)

tended to increase. After 24 and 48 hour storage periods, test samples appeared to be more stable than either control sample. The data suggest that sodium benzoate at concentrations of 0.3% and 0.8% showed a preservative effect up to a 48 hour storage period and sample stability appeared significantly better ($p < 0.05$) than the 22°C control sample; however, there is no significant difference when compared to the 4°C control sample. Sample stability was no longer evident after storage for 72 hours for either the control or test samples. This indicated that sodium benzoate exhibited a preservative effect only for a 48 hr storage period but does not indicate an optimum concentration to be used.

D. Polyethylene Glycol

Evaluation of polyethylene glycol as a preservative is shown in Figure 5. The data in this figure show that the control sample stored at 22°C was more stable over a 72 hour period than the control stored at 4°C. Test samples that had polyethylene glycol concentrations of 10.0 mg/L and 0.1 mg/L appeared to be more stable than the control sample stored at 4°C but showed no significant difference when compared to the control stored at 22°C even after a 72 hour storage period. Although the test sample containing 1.0 mg/L of polyethylene glycol did not appear to be as stable as the other two test samples, there was no significant difference in sample stability among the three concentrations of polyethylene glycol.

E. Other Preservatives

Sulfanilamide, ethylenediamine tetra-acetic acid (EDTA), glycerol and thioglycolate were also investigated as preservatives. Sulfanilamide failed to exhibit any preservative effect and glycerol stimulated growth within 24 hours. The raw data indicated that both EDTA and thioglycolate may show

favorable preservative capabilities, however, the statistical analyses were not completed for inclusion in this progress report.

The data obtained by the MPN procedure was not included in this report. A comparison of the capability of the MF and MPN procedures for recovery of coliform organisms from test samples containing a preservative showed that the MPN procedure was unreliable. Failure of the MPN procedure to detect coliform organisms was attributed to the bacteriostatic effect of the preservative introduced along with the sample into the MPN medium at the time of inoculation. This observation demonstrates that the MPN procedure may not be applicable for analyzing potable water samples for coliform occurrence when the samples contain a preservative.

Discussion

The need to stabilize the coliform density in potable water samples has been recognized for years. Most of the information relating to coliform density stability studies available in the literature is based upon the refrigeration of samples (2,3,4,5,6).

The data obtained for this study were difficult to analyze statistically. The major problem is that the variance in counts observed among replicates was, generally, as great as the variance in counts observed over time. The statistical approach used to analyze the data indicated that the coliform density of the control samples generally decreased during 48 hours storage regardless of storage temperature. However, the control stored at 4°C showed more stability during 48 hour storage than the control stored at 22°C. After a 72 hour storage period, the 4°C control generally continued to decline, whereas, the 22°C control showed more of a tendency to increase. Two of the preservatives, boric acid and sodium chloride, showed no stabilizing effect on

the coliform densities over any storage period. However, sodium chloride, at a concentration of 0.3%, stimulated growth significantly after the 48 and 72 hour storage periods.

Sodium benzoate appeared to function as a preservative during a 48 hour storage period. In fact, after storage periods of 24 and 48 hours, the test samples were significantly better ($p < 0.05$) than the control stored at 22°C. There was no significant difference in sample stability compared to the 4°C control sample. Increasing the storage period to 72 hours resulted in loss of sample stability. Additional studies will be conducted to determine the stabilizing ability of sodium benzoate at higher concentrations and for storage periods greater than 48 hours but less than 72 hours.

The studies conducted using polyethylene glycol resulted in an unexpected observation with regard to the control samples. Analyses of the data for the two control samples showed the control sample stored at 22°C was significantly more stable ($p \sim 0.06$) than the 4°C control sample. Previous investigators observed similar results when samples were stored at 22°C (3,4). However, the coliform density in test samples containing polyethylene glycol, at concentrations of 0.1 mg and 10.0 mg/L, were significantly more stable than the 4°C control and there was no significant difference in sample stability compared to the 22°C control. Therefore, it appears that polyethylene glycol may function as a preservative, but additional studies must be conducted to obtain data to determine which preservative, polyethylene glycol or sodium benzoate, is better.

Tentative Conclusions

1. Sodium benzoate and polyethylene glycol appear to stabilize the initial coliform density in test samples for 48 hours at 22°C.

2. Boric acid, sodium chloride, sulfanilamide and glycerol do not appear to function as preservatives.

3. The MPN procedure may not be applicable to analyzing potable water samples which contain preservatives.

Additional Research Needs

1. Investigate effect of candidate preservatives at a higher temperature and under conditions in which storage temperature fluctuates.

2. Determine sample stability when the initial coliform density is less than 10/100 mL and the sample contains variable levels of heterotrophic bacteria.

3. Explore the additive effect on coliform stability when a mixture of various concentrations of two or more preservatives are used.

4. Field evaluate this sample preservative concept on samples collected from various distribution systems.

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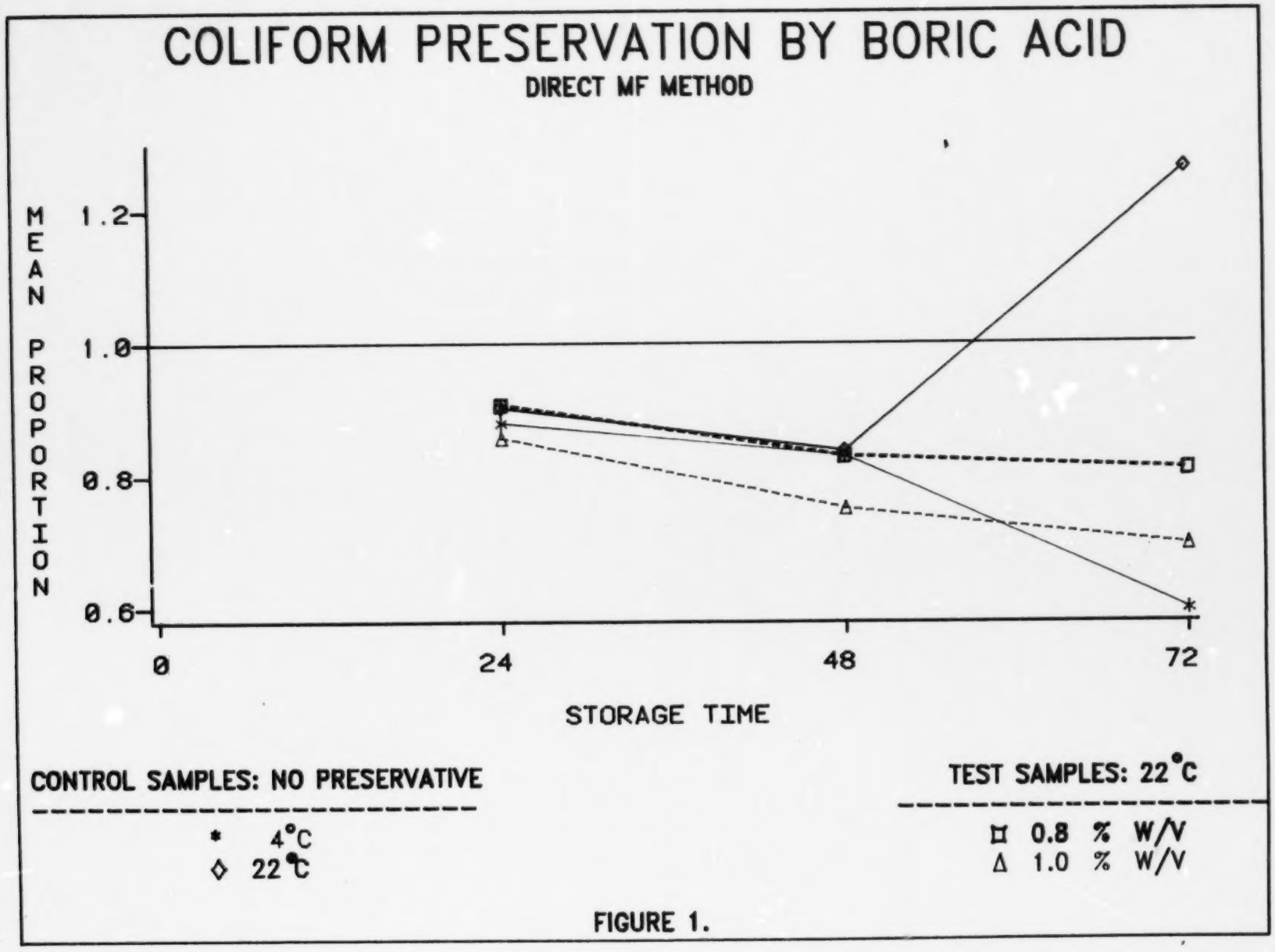
COLIFORM PRESERVATION BY BORIC ACID
DIRECT MF METHOD
MEAN PROPORTION
1.2
1.0
0.8
0.6
0
24
48
72
STORAGE TIME
CONTROL SAMPLES: NO PRESERVATIVE
* 4°C
◇ 22°C
TEST SAMPLES: 22°C
□ 0.8 % W/V
Δ 1.0 % W/V

FIGURE 1.

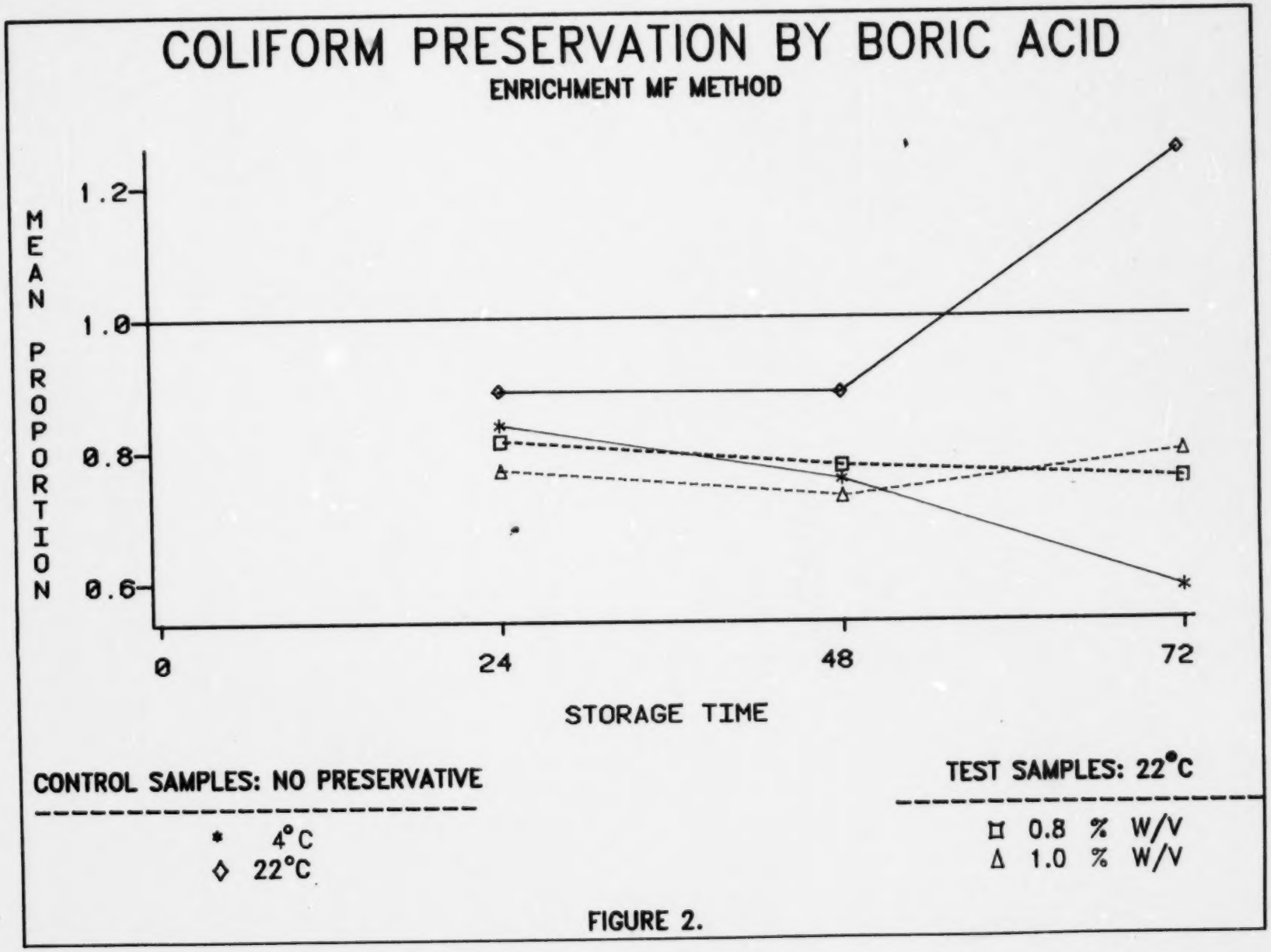
COLIFORM PRESERVATION BY BORIC ACID
ENRICHMENT MF METHOD
MEAN PROPORTION
1.2
1.0
0.8
0.6
0
24
48
72
STORAGE TIME
CONTROL SAMPLES: NO PRESERVATIVE
* 4°C
◇ 22°C
TEST SAMPLES: 22°C
□ 0.8 % W/V
Δ 1.0 % W/V

FIGURE 2.

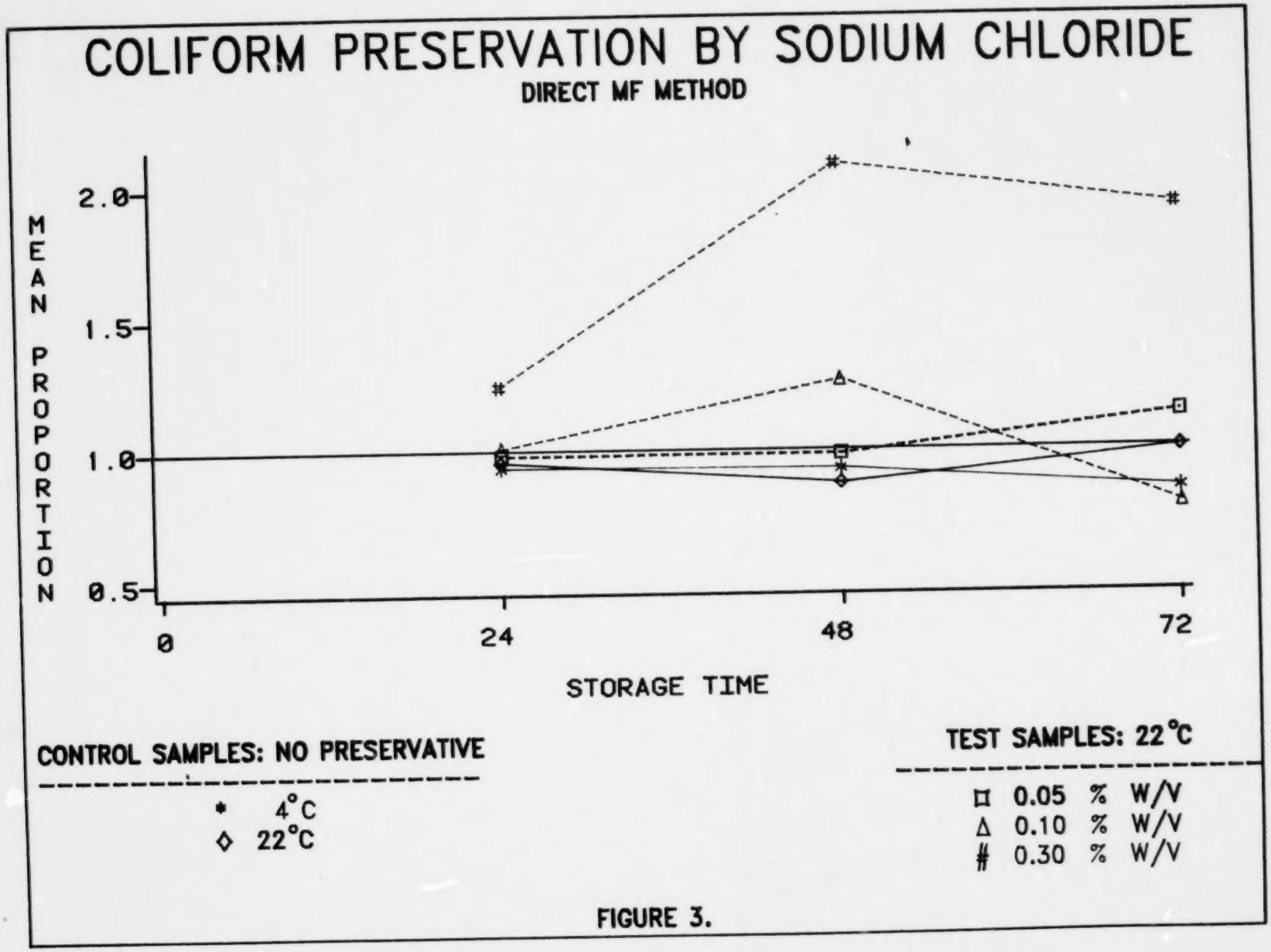
COLIFORM PRESERVATION BY SODIUM CHLORIDE
DIRECT MF METHOD
MEAN PROPORTION
2.0
1.5
1.0
0.5
0
24
48
72
STORAGE TIME
CONTROL SAMPLES: NO PRESERVATIVE
* 4°C
◇ 22°C
TEST SAMPLES: 22°C
□ 0.05 % W/V
△ 0.10 % W/V
0.30 % W/V

FIGURE 3.

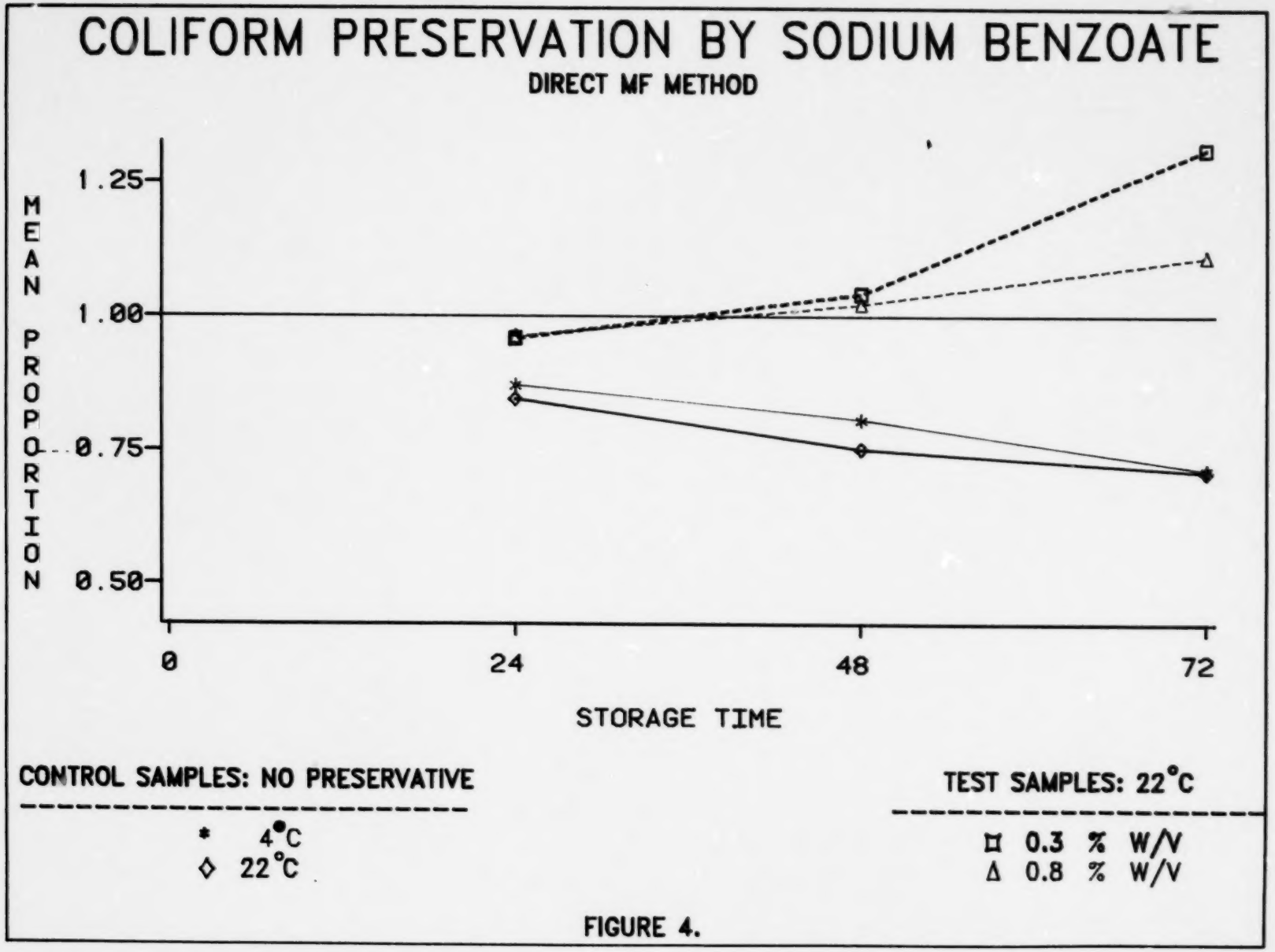
COLIFORM PRESERVATION BY SODIUM BENZOATE
DIRECT MF METHOD
MEAN PROPORTION
1.25
1.00
0.75
0.50
0
24
48
72
STORAGE TIME
CONTROL SAMPLES: NO PRESERVATIVE
* 4°C
◇ 22°C
TEST SAMPLES: 22°C
□ 0.3 % W/V
Δ 0.8 % W/V

FIGURE 4.

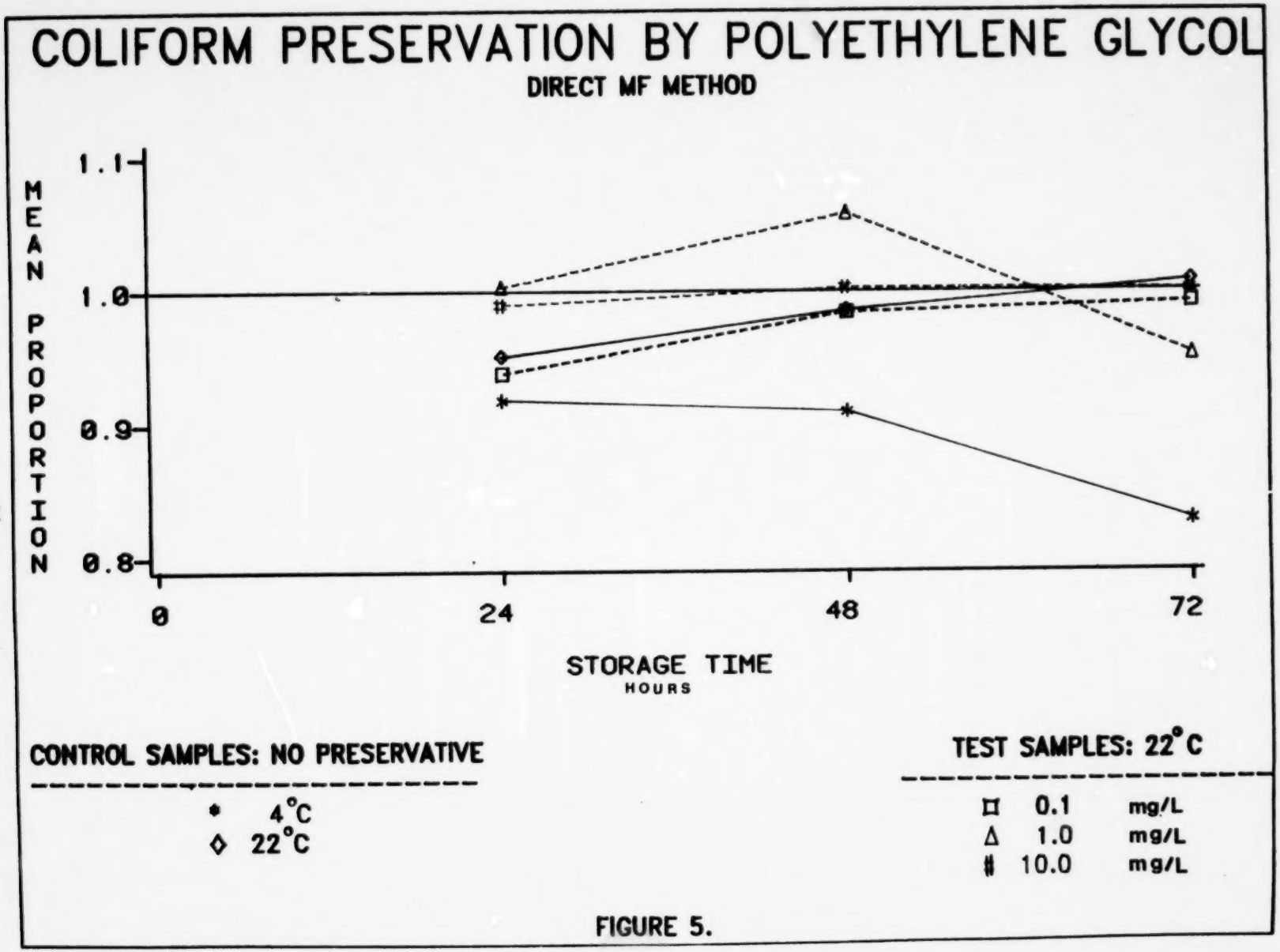
COLIFORM PRESERVATION BY POLYETHYLENE GLYCOL
DIRECT MF METHOD
MEAN PROPORTION
1.1
1.0
0.9
0.8
0
24
48
72
STORAGE TIME
HOURS
CONTROL SAMPLES: NO PRESERVATIVE
4°C
22°C
TEST SAMPLES: 22°C
0.1 mg/L
1.0 mg/L
10.0 mg/L

FIGURE 5.